

Transcriptome changes induced by docetaxel in human mammary cell lines expressing different levels of ERBB2

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Abstract. The taxane docetaxel is currently the most effective chemotherapeutic drug for the treatment of advanced breast cancer. However, a considerable proportion of breast cancer patients do not respond positively to docetaxel. The mechanisms of docetaxel resistance are poorly understood. Overexpression of *ERBB2* occurs in 15-30% of breast tumors and is associated with chemoresistance to a variety of anticancer drugs. In the present study, we sought to identify genes involved in *ERBB2*-mediated chemoresistance to docetaxel. We generated SAGE libraries from two human mammary cell lines expressing basal (HB4a) and high (C5.2) levels of *ERBB2* before and after intensive exposure to docetaxel and identified potential *ERBB2* target genes implicated in a variety of cellular processes including cell proliferation, cell adhesion, apoptosis and cytoskeleton organization. Comparison of the transcriptome of the cell lines before and after docetaxel exposure revealed substantially different expression patterns. Twenty-one differentially expressed genes between HB4a and C5.2 cell lines, before and after docetaxel treatment, were further analyzed by qPCR. The alterations in the expression patterns in HB4a and C5.2 cell lines in response to docetaxel treatment observed by SAGE analysis were confirmed by qPCR for the majority of the genes analyzed. Our study provides a comprehensive view of the expression changes induced in two human mammary cells expressing different levels of *ERBB2* in response to docetaxel that could contribute to the elucidation of the mechanisms involved in *ERBB2*-mediated chemoresistance in breast cancer.

Introduction

Breast cancer is the most commonly diagnosed malignancy and the second leading cause of mortality related to cancer in women living in Western countries (1). In spite of the advances in our knowledge of the numerous genetic and epigenetic changes associated with human breast carcinogenesis, chemotherapy is still the choice for patients with advanced breast tumors. A major clinical problem associated with fatality of advanced breast cancer patients is the intrinsic or acquired resistance to chemotherapy that leads to a high recurrence rate and reduced overall survival (2). To date, there are no clinically useful predictive markers to distinguish patients who are likely to respond effectively to a chemotherapeutic regimen.

The taxanes paclitaxel and docetaxel are anti-microtubule agents that exert their anti-proliferative effects by binding to the β subunit of tubulin, a key component of microtubules, dynamic polymers that play various roles in cell physiology, including cell division (3). Taxane binding prevents microtubule depolymerization (4), which disrupts normal mitotic spindle formation resulting in an inhibition of cell division at the G2-M phase of the cell cycle (5,6).

Docetaxel is currently the most effective chemotherapeutic drug for the treatment of advanced breast cancer (7). However, a considerable proportion of breast cancer patients do not respond positively to docetaxel, but instead suffer from side effects. To date, the mechanisms of docetaxel resistance are poorly understood. Intrinsic or acquired docetaxel resistance has been associated with altered expression of several genes: 1) overexpression of P-glycoprotein (8,9); 2) altered expression of β -tubulin isoforms (10); 3) reduced expression of p27 (11); 4) overexpression of CYP3A4 (12); 5) overexpression of the BRCA2 gene (13); 6) overexpression of CCT5, RGS3 and YKT6 genes (14); 7) amplification of 7q21 and loss of 10q chromosome region (9); and 8) overexpression of ERBB2 (15,16). However, the clinical significance of these proposed predictive factors remains to be established.

Overexpression of ERBB2, a member of the transmembrane receptor tyrosine kinase family, occurs in 15-30% of invasive breast tumors (17,18) and is associated with increased aggressiveness and chemoresistance to a variety of anticancer drugs, including docetaxel (15,16,19). ERBB2 is

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proving to be an excellent target for therapeutic approaches in breast cancer. Indeed, the recombinant humanized monoclonal anti-ERBB2 antibody, Herceptin, has been shown to have significant therapeutic effects in patients with ERBB2-positive breast cancer, particularly when combined with anticancer drugs such as paclitaxel and docetaxel (20).

Investigation of docetaxel modulated gene expression may lead to the identification of biomarkers of docetaxel clinical activity, which may be useful in overcoming resistance. In the present study, we sought to identify genes involved in ERBB2-mediated chemoresistance to docetaxel, and examined differential gene expression from SAGE libraries of two human mammary luminal cell lines expressing different levels of ERBB2 before and after intensive exposure to docetaxel.

Materials and methods

Cell culture and treatment. The mammary cell lines, HB4a and C5.2, were kindly provided by Dr Michael O'Hare from the Ludwig Institute for Cancer Research, London, UK. The C5.2 cell line was established by transfection of parental normal mammary luminal epithelial HB4a cells with full-length normal human ERBB2 cDNA derived from the breast cancer cell line BT474 (21). Cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml hydrocortisone and insulin in a 5% CO₂ humidifier incubator at 37°C. The breast carcinoma cell line, SKBR3, was kindly provided by Dr Anamaria Aranha Camargo (Ludwig Institute for Cancer Research, São Paulo, Brazil). SKBR3 cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin in a 5% CO₂ humidifier incubator at 37°C. Docetaxel (Taxotere) was obtained from Aventis Pharmaceuticals Inc., Bridgewater, NJ and diluted in ethanol at 10⁻² M and stored at -20°C. The cells were made resistant to docetaxel by short-term *in vitro* exposure to 5 nM docetaxel for 1 h followed by treatment with increasing concentrations of docetaxel (0.01-1 µM for C5.2 cells and 0.01-0.250 µM for HB4a cell line) each 24 h.

RNA extraction. After the treatments, the cells were washed twice with PBS and harvested. Total RNA was extracted from cultured cells by the guanidine isothiocyanate method as previously described (22). The quality of the RNA samples was determined by 1% agarose gel electrophoresis and ethidium bromide staining. All RNA samples were treated with DNaseI for 1 h at 37°C to eliminate genomic DNA contamination.

Serial analysis of gene expression (SAGE). Total RNA (25 µg) isolated from HB4a and C5.2 cell lines before and after intensive exposure to docetaxel were used to generate SAGE libraries. SAGE was carried out using the I-SAGE kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol based on the original SAGE method (23). Tag frequency tables were obtained from sequences by SAGETM analysis software, with minimum tag count set to one, maximum ditag length set to 28, and other parameters set as default. The annotation was based on SAGEmap (

Figure 1. Venn diagrams of the number of differentially expressed genes in SAGE libraries generated from HB4a and C5.2 cell lines before and after intensive exposure to docetaxel. Using a cutoff of ≥ 4 fold changes we identified 411 genes more expressed in HB4a and 282 genes more expressed in C5.2 cells after treatment with docetaxel.

ncbi.nlm.nih.gov/SAGE) and CGAP SAGE Genie (<http://cgap.ncbi.nlm.nih.gov/SAGE>). Statistical analysis was carried out with the H2G (hyper- and hypo-expressed genes) software (<http://www.gdm.fmrp.usp.br/h2g>) for the comparison of two SAGE libraries. H2G was also used to perform the normalization of compared libraries using the library with the highest number of tags as reference. Functional categorization of the differentially expressed genes was performed using GO (www.geneontology.org). Network analysis of the SAGE data was performed using the Ingenuity pathway analysis software (Ingenuity Systems Inc.).

Transfection of cells with siRNA. C5.2 cells were cultured in 6-well plates with complete medium until they reach 50% confluence. Medium was then replaced by medium without serum and antibiotics and cells were transfected with 50 nM of siERBB2 or siControl (siC) in RPMI-1640 using Lipofectamine reagent (Invitrogen) and OptiMEM (Invitrogen) according to the manufacturer's instructions. After 6 h, 10% FCS, insulin and hydrocortisone were added to the cultures. siRNA transfected cells were grown in complete medium for 72 h, and cells were then harvested for total RNA and protein extraction.

Western blotting. Untransfected, siControl (siC) and ERBB2 siRNA (siERBB2) transfected C5.2 cells were washed 3 times with PBS, harvested by scraping and collected in PBS. The cell suspension was centrifuged at 2,000 rpm for 2 min. The pellet was dissolved in lysis buffer (50 mM Na pyrophosphate, 50 mM NaF, 5 mM NaCl, 5 mM PMSF, 100 mM Na₃VO₄) and centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was collected and stored at -70°C. Protein concentration was measured using Bradford reagent (Pierce, Rockford, IL). Aliquots (15 µg of protein) from each sample were separated by electrophoresis on a 7.5% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane at 100 V for 2 h using transfer buffer (24 mM Tris, 193 mM glycine, 20% methanol). The membranes were blocked in 5% non-fat dried milk in TBS containing 0.1% Tween-20 (TBSt) for 1 h at room temperature and then incubated overnight in a cold room with 3% albumin containing anti-ERBB2 antibody. Blots were washed 3 times

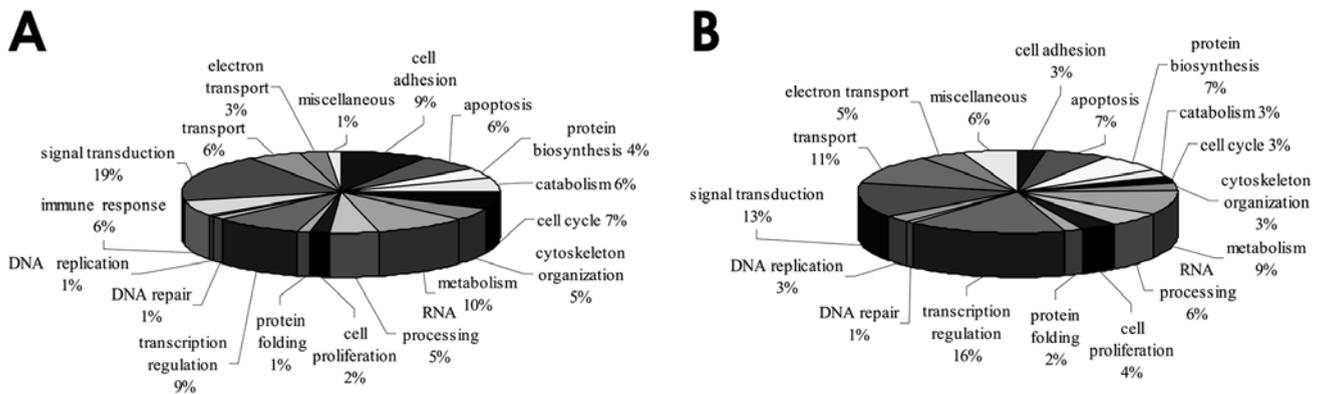


Figure 2. Distribution of the highly expressed transcripts from HB4a (A) and C5.2 (B) cell lines after docetaxel treatment after classification into functional categories using Gene Ontology nomenclature.

with TBSt and incubated with anti- β -actin antibody for 1 h. After three washes in TBSt for 10 min blots were incubated with peroxidase-conjugated anti-mouse secondary antibody (all antibodies were purchased from Chemicon International, Billerica, MA and diluted 1:1000). Following three washes, labeled proteins were detected using Supersignal West Pico (Pierce).

Quantitative real-time PCR (qPCR). Total RNA (10 μ g) was reverse transcribed using the High capacity cDNA archive kit (Applied Biosystems). PCR amplification was performed using an Applied Biosystems PRISM 5700 sequence detector and the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) following the manufacturer's recommendations. qPCR reactions were carried out under the following conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 55°C for 1 min. Gene expression was normalized against GAPDH expression as an internal control. The results were expressed as *n*-fold differences in gene expression relative to the calibrator sample. The relative expression was calculated by $2^{-\Delta\Delta CT}$ (CT = fluorescence threshold value; ΔCT = CT of the target gene - CT of the reference gene (GAPDH); $\Delta\Delta CT$ = ΔCT of the target sample - ΔCT of the reference sample).

Results

The aim of this study was to identify potential candidate genes involved in docetaxel resistance mediated by ERBB2 amplification and/or overexpression. We employed SAGE technique to examine the gene expression profile from two normal mammary cell lines, HB4a and C5.2, which express different levels of ERBB2 before and after intensive exposure to docetaxel.

A total of 307,506 tags was generated by sequencing from HB4a (81,684 tags; 30,854 unique tags), HB4a treated with docetaxel (70,715; 31,546 unique tags), C5.2 (79,948 tags; 30,568 unique tags) and C5.2 treated with docetaxel (75,159; 31,293 unique tags). Analysis of the expression profiles of HB4a and C5.2 cells prior to docetaxel treatment was previously published (24).

Comparison of the transcriptome of the cell lines prior to and after docetaxel exposure revealed substantially different

expression patterns. Alterations in expression patterns of the top 50 previously identified genes as more expressed in HB4a and C5.2 cells (24) in response to docetaxel treatment are shown in Tables I and II. Using a cutoff of ≥ 4 -fold changes in examining the differences in expression between the transcripts from HB4a and C5.2 libraries, we detected increased expression of 411 distinct genes in HB4a and 282 distinct genes in C5.2 treated with docetaxel. Among the genes differentially expressed between HB4a and C5.2 treated with docetaxel, only a few genes were also found to be differentially expressed prior to treatment (see Venn diagrams in Fig. 1). A considerably higher number of ESTs and hypothetical proteins were identified in the cell line C5.2, which express high levels of *ERBB2*, after docetaxel exposure. Functional characterization of the genes differentially expressed between HB4a and C5.2 cell lines prior to docetaxel treatment was previously reported (24).

HB4a cells exposed to intense docetaxel treatment showed higher percentage of genes involved in apoptosis (HB4a+Doc, 6% vs. HB4a-Doc, 1%), cell adhesion (HB4a+Doc 9% vs. HB4a-Doc, 2%) and immune response (HB4a+Doc, 6% vs. HB4a-Doc, 2%) compared to HB4a cells before docetaxel treatment (Fig. 2). The genes differentially expressed between HB4a and C5.2 cell lines were also subjected to Ingenuity pathway analysis (www.ingenuity.com). Analysis of the genes down- and up-regulated in the cell lines before and after docetaxel treatment confirmed that *ERBB2* is a key molecule associated with the modulation of gene expression in our system (Figs. 3 and 4).

Based on tag abundance and functional annotation, 21 of the differentially expressed genes in the HB4a and C5.2 cells before and after intensive exposure to docetaxel were selected for validation by qPCR. This panel included 14 up-regulated genes (MATR3, ATAD4, HDGF, CENPH, LGALS1, AES, ANP32B, LEMD3, CAV1, BTG1, COQ10B, DBNDD2, NDRG1 and C6orf115) and 7 down-regulated genes (ACTN1, ETFB, RUVBL1, DUSP1, SPARC, TPM1 and SFRP1) in C5.2 compared to HB4a cells, before intensive exposure to docetaxel. This first validation was performed using the cDNA samples previously used for the construction of the SAGE libraries. The alterations in the expression patterns between HB4a and C5.2 cell lines in response to docetaxel treatment as assessed by qPCR were consistent with the

Table I. Gene expression changes of the top 50 genes down-regulated in C5.2 versus HB4a mammary cells in response to docetaxel treatment.

TAG	UniGene	Description (locus name)	HB4a ^a	C5.2 ^a	HB4T	C5.2T
AAAATATTTT	Hs.509765	Actinin, α 1 (ACTN1)	23	1	11	11
AAAGCCAAGA	Hs.654553	Electron-transfer-flavoprotein, β polypeptide (ETFB)	18	0	19	2
AAGTGAGATG	Hs.272822	RuvB-like 1 (<i>E. coli</i>) (RUVBL1)	15	0	2	1
AAGAGTTACG	Hs.55041	Mitochondrial ribosomal protein L2 (MRPL2)	15	1	5	3
AGAAATGTAT	Hs.282113	SNF1-like kinase (SNF1LK)	14	1	11	5
AGTGTGATAC	Hs.118820	Hypothetical protein BC007882 (LOC152217)	14	1	3	7
AGGAAAAGAT	Hs.523829	Polymerase (DNA-directed), δ 4 (POLD4)	12	1	15	3
CTTGACATAC	Hs.171695	Dual specificity phosphatase 1 (DUSP1)	12	1	13	4
AGGGCTACGG	BF571807	EST	12	1	2	0
CCAGCCCAGC	Hs.29802	Slit homolog 2 (<i>Drosophila</i>) (SLIT2)	11	1	4	3
ACTGCGAGGA	Hs.110477	Dolichyl-phosphate mannosyltransferase polypeptide 3 (DPM3)	10	0	1	7
ATGTCTTTTC	Hs.462998	Insulin-like growth factor binding protein 4 (IGFBP4)	10	1	7	5
GGCCTTTTTT	Hs.75307	H1 histone family, member X (H1FX)	10	1	3	3
CTACGTGCTC	Hs.521056	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit f, isoform 2 (ATP5J2)	9	1	1	0
AATTTGCAAC	Hs.420272	H2A histone family, member Y (H2AFY)	9	1	6	2
CTTGATTCCC	Hs.518374	Quiescin Q6 sulfhydryl oxidase 1 (QSOX1)	9	1	5	6
CACTTGAAAA	Hs.7753	Calumenin (CALU)	9	1	6	1
TTGGGAGCAG	Hs.445403	Isoleucine-tRNA synthetase (IARS)	9	1	7	3
ACGATTGATG	Hs.528320	Apolipoprotein A-I binding protein (APOA1BP)	9	1	3	4
AAAATAAAAA	Hs.517373	Protoporphyrinogen oxidase (PPOX)	9	1	2	6
CGTTCCTGCG	Hs.504609	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein (ID1)	9	1	3	1
ATGTGAAGAG	Hs.111779	Secreted protein, acidic, cysteine-rich (osteonectin) (SPARC)	141	17	22	18
GATCCCAAAC	BF686592	EST	8	0	5	3
TTCTTGCTTA	Hs.425777	Ubiquitin-conjugating enzyme E2L 6 (UBE2L6)	8	0	20	2
AAGAAAACCTG	Hs.561815	Staufen, RNA binding protein, homolog 2 (<i>Drosophila</i>) (STAU2)	8	0	3	2
CTTGGTTCTC	Hs.501578	Mitochondrial GTPase 1 homolog (<i>S. cerevisiae</i>) (MTG1)	8	0	1	0
AATACCTCGT	Hs.414579	Scotin (SCOTIN)	8	0	11	3
GTTGCTGCCC	Hs.9234	Transmembrane protein 147 (TMEM147)	8	0	3	0
TTAAAGATTT	Hs.133892	Tropomyosin 1 (α) (TPM1)	8	0	4	4
GCCAAGATGC	Hs.515164	Growth arrest and DNA-damage-inducible, γ interacting protein 1 (GADD45GIP1)	16	2	4	5
AACATTCTAA	Hs.530436	Syntaxin binding protein 3 (STXBP3)	8	1	4	4
TATCAATATT	Hs.213424	Secreted frizzled-related protein 1 (SFRP1)	8	1	0	1
AAGGATCCCC	Hs.558499	CD320 molecule (CD320)	8	1	1	2
CTCTAGAACC	Hs.292579	Phosphatidylserine synthase 1 (PTDSS1)	8	1	2	3
GCATCTGTTT	Hs.520189	ELOVL family member 5, elongation of long chain fatty acids (ELOVL5)	8	1	2	2
AACCAGAGGT	Hs.529280	Anaphase promoting complex subunit 7 (ANAPC7)	8	1	4	2
TAACAAAGAA	Hs.632540	Protein kinase, interferon-inducible double-stranded RNA dependent activator (PRKRA)	8	1	1	10
GAATAAAATA	Hs.269592	Coiled-coil and C2 domain containing 1A (CC2D1A)	8	1	8	5
GACTCTTCAG	Hs.534293	Serine (or cysteine) proteinase inhibitor, clade A (α -1 antiproteinase, antitrypsin), member 3 (SERPINA3)	8	1	0	10
TGATTTATTC	Hs.465870	Kelch-like ECH-associated protein 1 (KEAP1)	8	1	5	3
ATGGCAACAG	Hs.505654	Integrin, α 5 (fibronectin receptor, α polypeptide) (ITGA5)	8	1	5	2
TGGCTTAAAT	Hs.433213	Hypoxia-inducible protein 2 (HIG2)	8	1	0	0
AAAGTGCATC	Hs.193326	Fibroblast growth factor receptor-like 1 (FGFRL1)	8	1	4	2
GACCTCCTGC	Hs.502872	Mitogen-activated protein kinase kinase kinase 11 (MAP3K11)	8	1	2	1
GATTTCAACC	Hs.371698	RNA export 1 homolog (<i>S. pombe</i>) (RAE1)	7	0	1	2
GTGGGGGGAG	Hs.446374	Prefoldin subunit 6 (PFDN6)	7	0	4	4
AATGACTGAA	Hs.93659	Protein disulfide isomerase family A, member 4 (PDIA4)	7	0	4	4
ATGCGGGAGA	Hs.534521	Transmembrane protein 54 (TMEM54)	7	0	2	0
CACTCTATCC	Hs.258551	Aspartyl aminopeptidase (DNPEP)	7	0	2	0
TACAAACCTG	Hs.513626	Metallothionein 1F (functional) (MT1F)	7	0	4	1

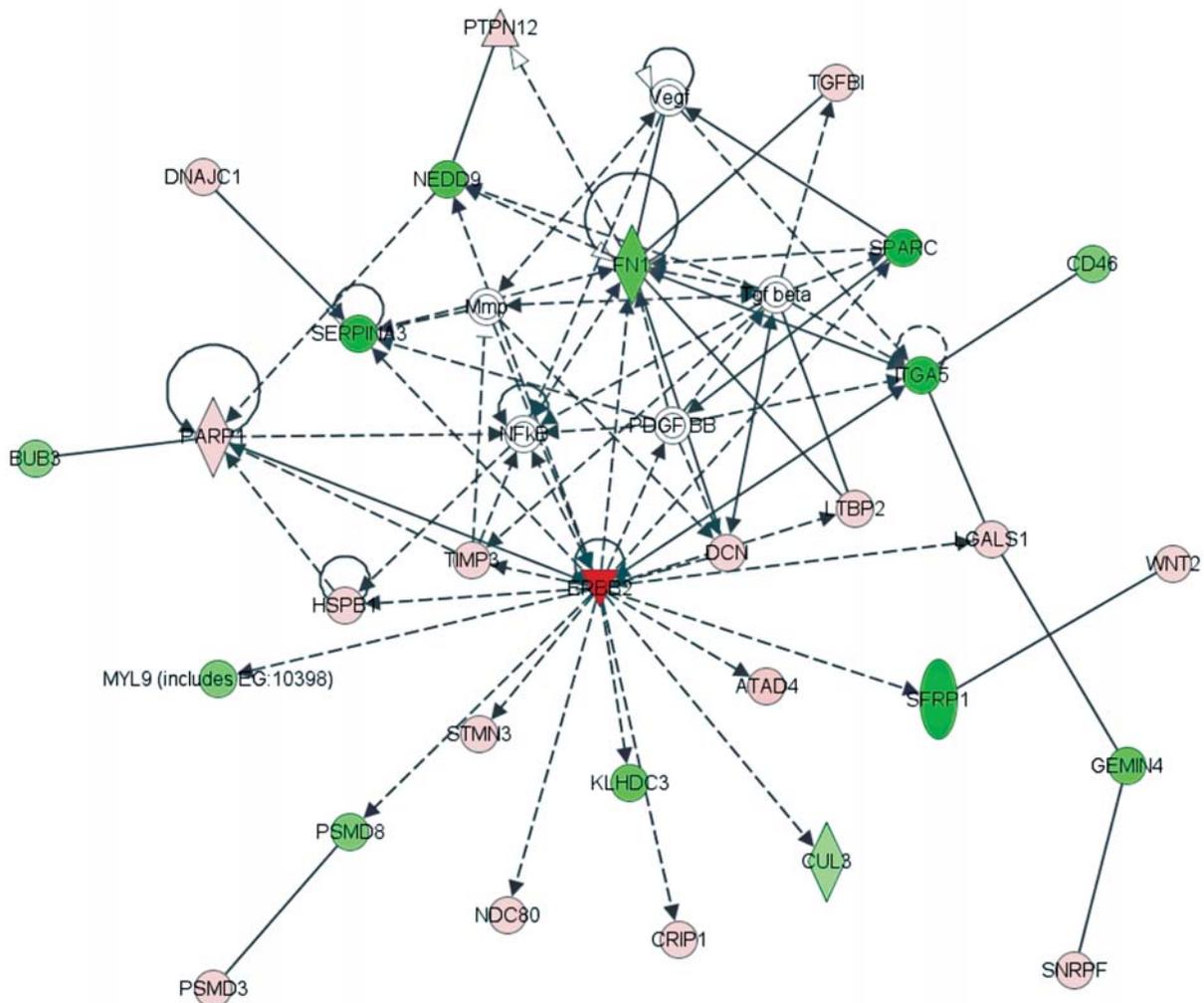
T, treated with docetaxel. ^ados Santos *et al.* (24).

Table II. Gene expression changes of the top 50 genes up-regulated in C5.2 versus HB4a mammary cells in response to docetaxel treatment.

TAG	UniGene	Description (locus name)	HB4a ^a	C5.2 ^a	HB4T	C5.2T
CTGTGTTTAG	Hs.268939	Matrin 3 (MATR3)	0	230	0	451
CCAGGAACCT	Hs.446352	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) (ERBB2)	0	85	0	44
GCAGGTTCCC	AI624297	EST	1	19	0	17
TAAGTAAAGT	Hs.368260	ATPase family, AAA domain containing (ATAD4)	0	16	0	7
CTATTTAGGG	Hs.7736	Mitochondrial ribosomal protein L27 (MRPL27)	0	16	3	18
TCTTCTCCCT	Hs.506748	Hepatoma-derived growth factor (high-mobility group protein 1-like) (HDGF)	1	14	17	6
CCCAGCTAAT	Hs.631967	Centromere protein H (CENPH)	2	25	4	7
TGATTGGTGG	Hs.74615	Platelet-derived growth factor receptor, α polypeptide (PDGFRA)	1	12	1	7
GCCCCAATA	Hs.445351	Lectin, galactoside-binding, soluble, 1 (galectin 1) (LGALS1)	2	23	6	8
TACCATCGAT	BI226498	EST	1	11	4	2
GGCAGAGACC	Hs.574492	Nucleoporin 62 kDa (NUP62)	1	11	3	3
GCTCTGCCTC	Hs.252549	Cathepsin Z (CTSZ)	0	10	1	1
CACGCAATGC	Hs.515053	Amino-terminal enhancer of split (AES)	1	10	7	1
GATTTTGTAG	Hs.494604	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member B (ANP32B)	2	18	8	8
TAGCTTCCTT	Hs.44276	Homeo box C10 (HOXC10)	1	9	2	5
TAAACATTGT	Hs.505905	LEM domain containing 3 (LEMD3)	1	9	1	2
GTAGACACCT	Hs.666222	Ribosomal protein L7 antisense mRNA, partial sequence	2	17	0	9
ATGGCAGGAG	Hs.617352	Homo sapiens, clone IMAGE:4715570	0	8	1	9
TAAATACAGT	Hs.503043	Carnitine palmitoyltransferase 1A (liver) (CPT1A)	0	8	4	2
TGTATCACTG	BF932124	EST	0	8	0	4
CATTGTAATA	BE871060	EST	0	8	1	10
CCTAAGGCTA	Hs.108371	E2F transcription factor 4, p107/p130-binding (E2F4)	0	8	4	5
TGGATCAACC	Hs.74034	Caveolin 1, caveolae protein, 22 kDa (CAV1)	0	8	2	0
CAGGAACCTG	Hs.572315	Transcribed locus	0	8	x	x
TCACAGCTGT	Hs.255935	B-cell translocation gene 1, anti-proliferative (BTG1)	2	16	10	9
CTACCAGGCC	Hs.54457	CD81 molecule (CD81)	1	8	3	2
TCTCAATTCT	Hs.690198	Cell division cycle 42 (GTP binding protein, 25 kDa) (CDC42)	1	8	5	3
GCTTTCATTG	Hs.708366	Nuclear casein kinase and cyclin-dependent kinase substrate 1 (NUCKS1)	1	8	2	4
TTTTGATAAA	Hs.388927	YY1 transcription factor (YY1)	1	8	7	3
TCTGCTAAAG	Hs.593339	High-mobility group box 1 (HMGB1)	1	8	4	5
AAGATTGGG	AA846582	EST	1	8	0	6
TAAAGCAGATG	Hs.306425	Inhibitor of Bruton agammaglobulinemia tyrosine kinase (IBTK)	1	8	2	1
GCGAAACCCC	Hs.632547	Coenzyme Q10 homolog B (<i>S. cerevisiae</i>) (COQ10B)	1	8	24	17
CAGAATAATA	Hs.204475	HIV TAT specific factor 1 (HTATSF1)	1	8	1	4
GATTTGTAGC	BE279179	EST	2	15	7	7
ACCGCCTGTG	Hs.79625	Chromosome 20 open reading frame 149 (C20orf149)	2	15	8	4
TCCTTTGCAA	Hs.81892	Casein kinase 1, γ 1 (CSNK1G1)0	7	1	0	
CTACATAATA	AA903109	EST	0	7	1	2
CTGCCCTCCTT	Hs.655055	Dysbindin (dystrobrevin binding protein 1) domain containing 2 (DBNDD2)	0	7	9	12
GCTACAGGTA	Hs.349306	Ring finger protein 145 (RNF145)	0	7	2	2
TTCAAGAAAC	Hs.344151	Golgi autoantigen, golgin subfamily a, 4 (GOLGA4)	0	7	1	2
CTCATAAGAA	AV722184	EST	0	7	3	7
TTAGCTTGTT	BU074305	EST	0	7	1	6
CAGGATCCAG	Hs.546303	Suppression of tumorigenicity 13 (colon carcinoma) (Hsp70 interacting protein) (ST13)	0	7	1	2
GCAGATCGGG	Hs.646417	Similar to 60S ribosomal protein L6 (TAX-responsive enhancer element-binding protein 107) (TAXREB107) (Neoplasm-related protein C140) (LOC401725)	0	7	1	2
GCGGAGAGAG	BI524265	EST	3	21	8	9
AGTAGGTGGC	Hs.591538	Transcribed locus, weakly similar to NP_039504.1 [Schizosaccharomyces pombe]	2	14	4	12
CGAATAAAAT	Hs.139896	Macrophage erythroblast attacher (MAEA)	2	14	4	4
TACAATAAAC	Hs.507910	Progesterone receptor membrane component 2 (PGRMC2)	1	7	1	7
GTTGTGATGT	Hs.374477	Ewing sarcoma breakpoint region 1 (EWSR1)	1	7	5	2

T, treated with docetaxel. ^ados Santos *et al* (24).

Network 1 : Network ERBB2_HB4xC5.2 - 2008-05-13 08:40 AM : Network ERBB2_HB4xC5.2.xls



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Figure 3. Molecular interaction network showing an integrated view of the up- (red) and down-regulated (green) genes in C5.2 versus HB4a cells. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge. The intensity of node color indicates the degree of up- (red) or down- (green) regulation. Edges are displayed with various labels that describe the nature of the relationship between the nodes: — binding only; → acts on; curved black arrowhead, auto-regulation. Dotted edges represent an indirect interaction.

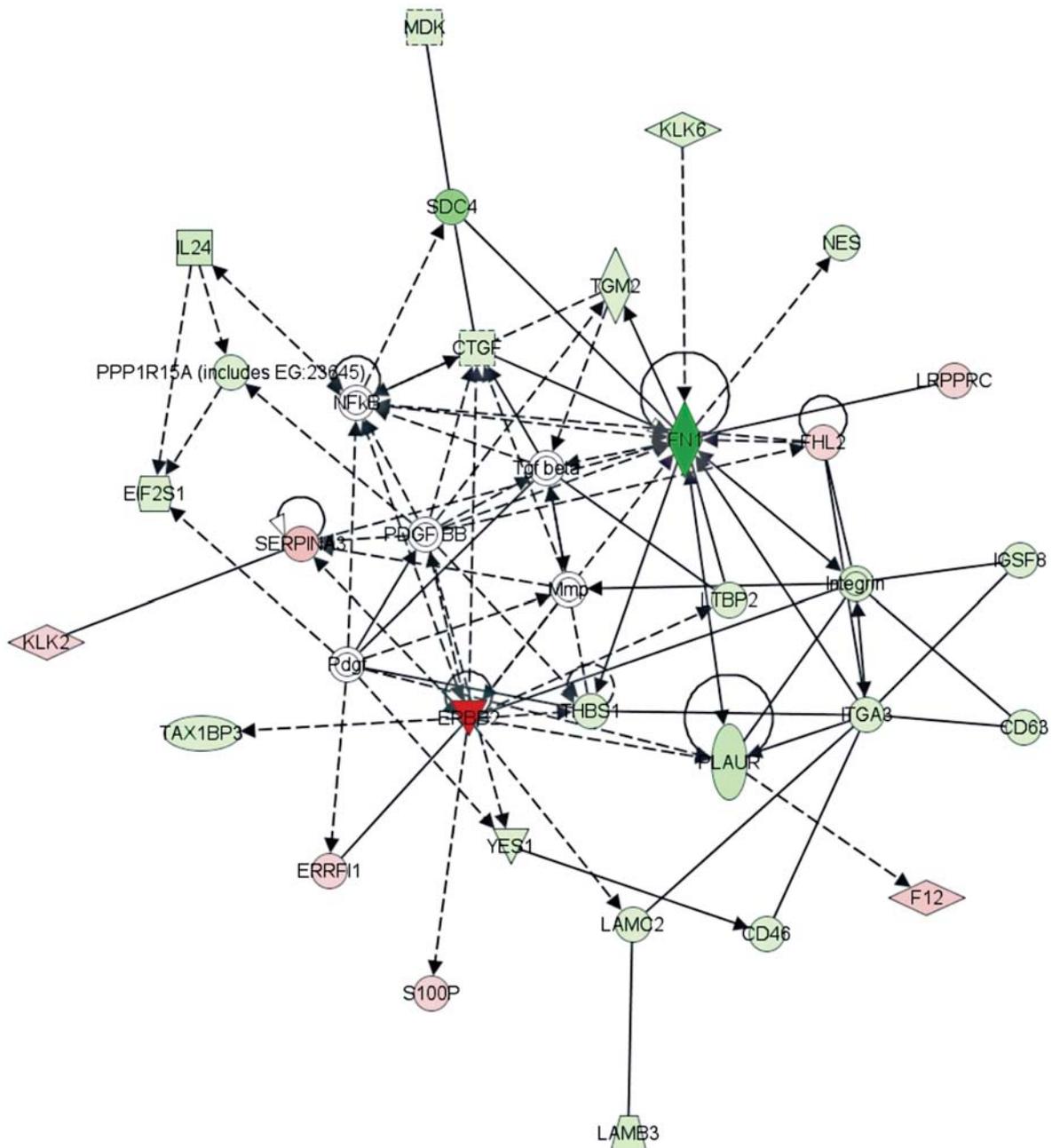
results generated by the SAGE method for the majority of genes analyzed. Representative results are shown in Fig. 5 (first two groups of bars).

Additional experiments were performed using cDNA samples from HB4a and C5.2 cells treated with 20 and 100 nM of docetaxel for 24h. Similar to the representative results shown in Fig. 5 (last groups of bars) for SPARC, RUVBL1, ATAD4 and CENPH, most tested genes exhibited similar down- and up-regulation as observed by SAGE analysis. SAGE and qPCR analysis showed decreased expression of SPARC (secreted protein, acidic, cysteine rich) and RUVBL1 (RuvB-like 1) in C5.2 cells compared to HB4a cells before treatment. Docetaxel treatment of C5.2 cells showed increased expression of SPARC and RUVBL1 leading to a similar expression levels of these genes in both cell lines (Fig. 5, last two groups of bars). In contrast, ATAD4 and CENPH transcripts, which were induced in C5.2 cells compared to HB4a cells prior to treatment showed no modulation (ATAD4)

or up-regulation (CENPH) in both cell lines after docetaxel treatment. Therefore, expression levels of ATAD4 and CENPH transcripts were similar before and after docetaxel treatment (Fig. 5).

To further confirm the expression pattern of SPARC, RUVBL1, ATAD4 and CENPH in mammary cell lines expressing different levels of ERBB2, we also examined the expression of these genes in the SKBR3 cell line. The results of three experiments shown in Fig. 6B, confirmed our previous results from SAGE and qPCR analysis, showing a down-regulation of SPARC and RUVBL1 transcripts in C5.2 and SKBR3 cells that overexpress ERBB2 compared to HB4a cells and an up-regulation of ATAD4 transcripts in both C5.2 and SKBR3 cells compared to HB4a cells. In contrast to our previous validation experiments, however, CENPH transcripts were shown to be down-regulated in both C5.2 and SKBR3 cell lines compared to HB4a cells.

Network 1: HB4TxC5.2T_ERBB2 - 2008-05-20 07:42 PM: HB4TxC5.2T_ERBB2.xls



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Figure 4. Molecular interaction network showing an integrated view of the up- (red) and down-regulated (green) genes in C5.2 versus HB4a cells after docetaxel treatment. Genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge. The intensity of node color indicates the degree of up- (red) or down- (green) regulation. Edges are displayed with various labels that describe the nature of the relationship between the nodes: — binding only; → acts on; curved black arrowhead, auto-regulation. Dotted edges represent an indirect interaction.

We further examined the impact of siRNA-mediated silencing of *ERBB2* on the expression of the differentially expressed genes. C5.2 cells transfected with siERBB2 showed a 60-70% decrease in *ERBB2* expression (Fig. 6A). siRNA-mediated *ERBB2* knockdown leads to decreased expression of *ATAD4* found to be up-regulated in C5.2 cells compared to HB4a cells by SAGE and qPCR analysis and increased expression of *CENPH* transcripts found to be down-regulated in C5.2 cells compared to HB4a cells by qPCR analysis.

Surprisingly, siERBB2 transfected cells displayed slightly decreased expression of *SPARC* and *RUVBL1* transcripts, which were found by both SAGE and qPCR analysis to be down-regulated in C5.2 cells (Fig. 6B).

Discussion

Clinical and experimental evidence indicates that amplification and/or overexpression of *ERBB2* is associated with docetaxel

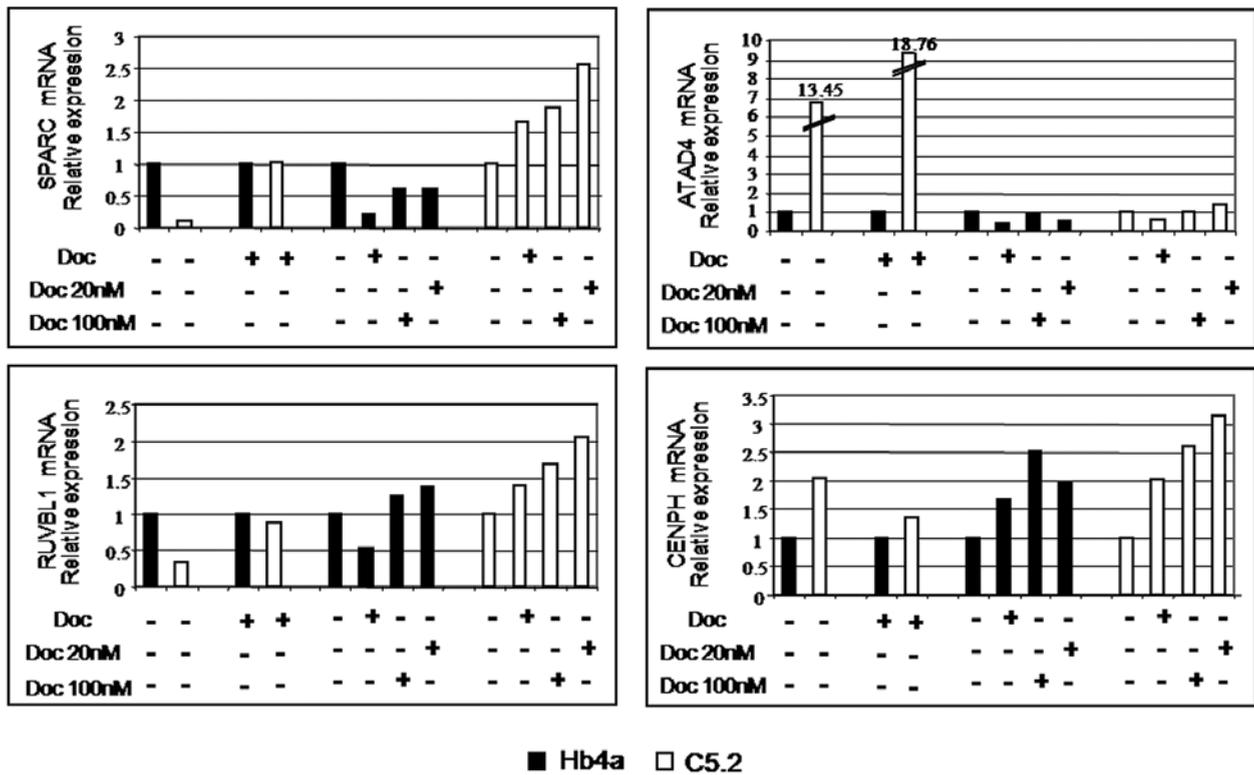


Figure 5. SPARC, RUVBL1, ATAD4, and CENPH expression in Hb4a and C5.2 cell lines before and after docetaxel treatment. The first two groups of bars represent the expression of genes between the HB4a and C5.2 cells before (Doc-) and after intensive exposure to docetaxel (Doc+). The last two groups of bars represent the expression of genes in Hb4a and C5.2 cell lines after treatment with 20 and 100 nM of docetaxel for 24 h.

resistance, but the predictive value of this association is not yet validated or fully understood (19). The aim of our study was to compare the changes in gene expression modulated by docetaxel in two human mammary cell lines expressing different levels of ERBB2 to identify potential candidate genes involved in the molecular mechanism of docetaxel sensitivity and resistance.

Comparative analysis between the mRNA expression profile of docetaxel-treated cells and untreated control cells revealed substantially different expression patterns in response to drug treatment. These differentially expressed genes were subsequently evaluated by their biological function (GO) and molecular interaction network analysis (Ingenuity Pathway Analysis program). In both cell lines, the highly expressed genes are associated with several biological processes. Both before and after docetaxel treatment we observed an over-representation of genes associated with signal transduction and cytoskeleton organization in HB4a cells compared to C5.2 cells and an over-representation of genes associated with cell proliferation, apoptosis and transport in C5.2 cells. These differences could be related to drug sensitivity. The most prominent networks were assembled around the ERBB2 oncogene, indicating that in C5.2 cells *ERBB2* is directly or indirectly influencing cell signaling.

Comparison of each cell line before and after intensive exposure to docetaxel reveals some significant changes in gene expression. HB4a cells, shown to be more sensitive to docetaxel, displayed a higher percentage of genes related to cell adhesion, apoptosis and immune response after intensive exposure to docetaxel compared to HB4a cells prior to treatment. Similar to the effects of many other chemo-

therapeutic agents, docetaxel exerts its cytotoxic effects by altering cell cycle and apoptosis regulation. Altered expression of genes involved in cell cycle and apoptosis control is observed in docetaxel-resistant human breast cancer cell lines and might be associated with acquired resistance to docetaxel in breast cancer cells (11). Depending on the concentration, docetaxel displays a biphasic cytotoxic effect in breast cancer cells, inducing aberrant mitosis followed by necrosis at a low concentration, and mitotic arrest followed by apoptosis at high concentration; furthermore, these two cytotoxic effects are related with specific gene expression profiles (25).

In addition, several studies have provided evidence that breast cancer cell lines and breast cancer patients exposed to paclitaxel and/or docetaxel show altered expression of several inflammatory cytokines and enzymes that might influence their clinical pharmacology and toxicology (26). Understanding the basis for the over-representation of induced genes related to apoptosis and immune response displayed by HB4a cells in response to docetaxel treatment compared to C5.2 cells that showed over-representation of cell proliferation and apoptosis-related genes and under-representation of immune related genes irrespective of docetaxel treatment, could contribute to elucidate the important role of the docetaxel response in breast cancer.

Notably, the intensive exposure to docetaxel resulted in a complex pattern of changes on gene expression profiles. Docetaxel was able to modulate the expression of several genes in both cell lines, indicating that docetaxel sensitivity and resistance involve the integration of many genes and biological pathways. Interesting, many genes, such as SPARC

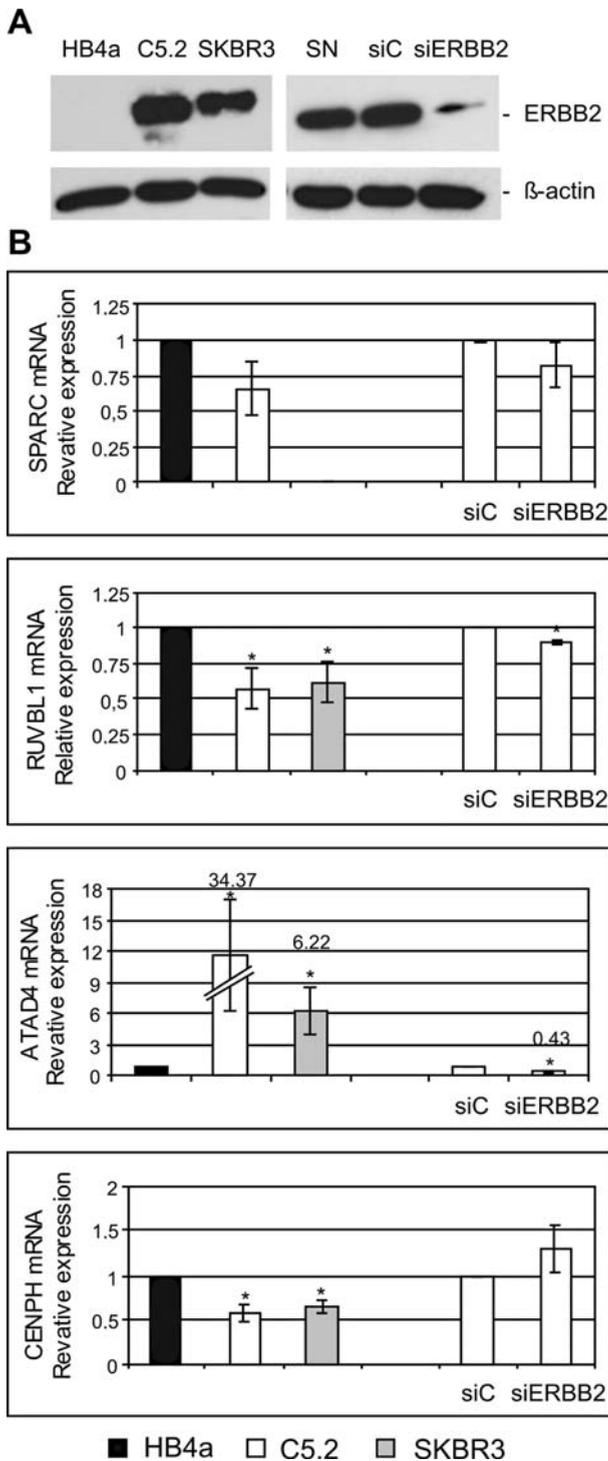


Figure 6. Effect of siRNA-mediated knockdown of ERBB2 on the expression of SPARC, RUVBL1, ATAD4, and CENPH in the C5.2 cells. (A) Western blotting shows total level of ERBB2 protein in Hb4a, C5.2 and SKBr3, and in C5.2 cells transfected with siControl or with siERBB2. Antibodies against β -actin were used as an endogenous control. (B) qPCR analysis of relative mRNA expression of SPARC, RUVBL1, ATAD4, and CENPH in Hb4a, C5.2 and SKBr3 cell lines, and in C5.2 cells transfected with siControl or with siERBB2. Results represent mean \pm SD of triplicate experiments. *Student's t-test, values of $p \leq 0.05$ were considered statistically significant.

and RUVBL1 that were differentially expressed between HB4a and C5.2 cells before drug treatment (24), were up- or down-regulated by docetaxel and showed a very similar

expression pattern after docetaxel exposure (data confirmed by qPCR), suggesting that these genes could play an important role in the sensitivity or resistance to docetaxel.

Although the precise molecular mechanism of docetaxel action is not fully understood, docetaxel is known to act as a microtubule-targeting agent that disrupts the dynamic process of microtubule assembly and disassembly, leading to cell cycle arrest and apoptosis (27). Some of the differentially expressed genes identified as modulated by docetaxel are related to cytoskeleton and spindle organization (RUVBL1) and cell to stroma adhesion (SPARC) that are important survival mechanisms associated with drug sensitivity (28,29).

The SPARC gene (secreted protein acid rich in cysteine; also termed osteonectin) encodes a matricellular protein that acts in various biological processes, including embryonic development, tissue remodeling, angiogenesis, proliferation, differentiation and cell migration and invasion (30). Altered expression of SPARC has been demonstrated in different types of tumors, however the role played by SPARC in cancer physiopathology is not fully understood and remains controversial (31,32).

In breast cancer patients, SPARC overexpression has been associated with a poor prognosis (33,34). Besides its role in ECM production, SPARC is also able to bind to many ECM components and growth factors to modulate their activity (30). Thus SPARC appears to not have a direct structural role, but instead acts as an important mediator of cell-matrix interaction and cell function, influencing the cell micro-environment and possibly drug availability (35,36). HB4a cells that are more sensitive to docetaxel exposure showed high expression levels of SPARC, which was down-regulated by intensive treatment with docetaxel. On the other hand, SPARC down-regulation was observed in both C5.2 and SKBR3 cells that express high levels of ERBB2, indicating that SPARC down-regulation could be associated with ERBB2 overexpression. However, the down-regulation of SPARC transcripts observed in C5.2 compared to HB4a cells could not be attributed solely to ERBB2 overexpression, as it was not affected by ERBB2 siRNA knockdown.

Tai *et al* (37) showed that low expression levels of SPARC are associated with decreased sensitivity to chemotherapy, and that restoration of SPARC expression, both *in vivo* and *in vitro*, is capable of reversing the resistant phenotype of colorectal cancer cells. These authors provided evidence that SPARC expression increases the sensitivity of colorectal cancer cells to radiation and 5-FU (Fluorouracil) due to modulation of the tumor microenvironment by SPARC, leading to increased apoptosis and blood vessel formation (37). Recent studies, demonstrated that the SPARC gene promoter is hypermethylated in colorectal tumors and that colorectal cells exposed to 5-aza-2'-desoxycytidine show increased expression of SPARC and higher sensitivity to 5-FU (38). These data suggest that SPARC could be a potential candidate marker for sensitivity to different chemotherapeutic drugs including docetaxel.

The RUVBL1 gene (also known as TIP49/Pontin52) localized on chromosome 3q21 encodes an ATP-dependent DNA helicase that shows homology to rat TIP49, a 49 kDa TATA box-binding interacting protein (39). RUVBL1 acts as an important co-factor of oncogenic transformation mediated

by c-myc (40) and β -catenin (41,42). In the present study, we found down-regulation of RUVBL1 transcripts in C5.2 cells that overexpress the ERBB2 oncogene compared to HB4a cells, but this expression pattern was not reversed by siRNA-mediated ERBB2 knockdown. However, RUVBL1 expression was modulated by docetaxel treatment and generated a similar expression pattern in both cell lines after docetaxel exposure, suggesting that RUVBL1 expression could be associated with docetaxel sensitivity and resistance.

Gartner *et al* (43) showed that RUVBL1 associates with tubulin and has an agonist effect on *in vitro* tubulin assembly, and RUVBL1-tubulin co-localize to the centrosomes. Fielding *et al* (44) showed that RUVBL1 is required for the centrosomal localization of ILK (integrin like kinase), one of the regulators of integrin mediated cell adhesion and cytoskeletal dynamics (45). In addition, Gartner *et al* (43) also showed that the extent of tubulin polymerization induced by the taxane paclitaxel is markedly increased in the presence of RUVBL1. Collectively, these results provide evidence that RUVBL1 could play a role in the sensitivity of cancer cells to anti-microtubule agents such as docetaxel and paclitaxel.

Although SPARC and RUVBL1 were found to be down-regulated in the mammary cell lines overexpressing ERBB2, our siRNA results showed that SPARC and RUVBL1 are not solely regulated by ERBB2 overexpression. On the other hand, our siRNA experiments helped to identify some genes that are either modulated (CENP-H) or not (ATAD4) by docetaxel treatment, and that are transcriptionally regulated by ERBB2 overexpression. The ATAD4 gene encodes a member of the ATPase family - AAA containing proteins, however its biological function is still unknown. In the present study, in corroboration with a previous study using cDNA microarray analysis (46) we found the ATAD4 gene transcripts to be up-regulated in C5.2 cells by SAGE and qPCR analysis. In addition, we demonstrated that the ATAD4 transcripts were significantly down-regulated after siRNA-mediated ERBB2 knockdown, indicating that ATAD4 is transcriptionally regulated by ERBB2 overexpression and could be associated with ERBB2 transformation. Further experimental and clinical studies will be required to better evaluate the role played by ATAD4 in breast cancer development and progression.

The CENP-H gene, located on chromosome 5q15.2, encodes a member of the centromere and kinetochore protein family that plays a fundamental role in centromere assembly and sister chromatid segregation (47,48). Chromosome missegregation is one of the main causes of aneuploidy, which is implicated in the tumorigenic process (49). CENP-H co-localizes with other members of the centromere and kinetochore protein family, such as CENP-A, CENP-B and CENP-C (47,50). Knockdown of CENP-H in a chicken cell line demonstrated that CENP-H is a necessary component for kinetochore complex formation (51). In human cells, RNAi knockdown of CENP-H results in severe multipolar spindles and misaligned chromosomes that, after a few cell cycles, lead to cell death (52). Increased CENP-H expression has been shown to play a role in the tumorigenic process of colorectal, oral squamous cell and nasopharyngeal carcinomas (53-55). To date, no study has reported the role of CENP-H in breast tumors. We first detected up-regulation of CENP-H transcripts in C5.2 cells by SAGE analysis, but qPCR results subsequently

showed that CENP-H transcripts are down-regulated in C5.2 and SKBR3 cell lines that overexpress ERBB2. However, as opposed to the ATAD4 gene, CENP-H transcripts were modulated by docetaxel treatment in both HB4a and C5.2 cells. Future studies will determine if altered expression of CENP-H also occurs in breast tumors and if this may have a predictive value for the docetaxel response.

The SAGE method allowed us to identify a large number of transcripts implicated in different cellular pathways that were up- or down-regulated by docetaxel in human mammary cell lines expressing different levels of the oncogenic protein erbB2. Whether these changes in expression are causally related to the transformation of the mammary cell lines by ERBB2 and/or implicated with chemotherapeutic resistance warrants future investigation in additional experimental and clinical studies. Further *in vitro* and *in vivo* studies are needed to elucidate the biological role of these genes and the possible association with sensitivity or resistance to docetaxel.

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